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Risks: Tumor Immunodiagnosis

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In a continuation project for the development of aquatic bioassays as alternatives for carcinogenicity and toxicity testing to assess human health risk, immunohistochemical techniques (IHC) for the diagnosis of carcinogen induced neoplasms and other proliferative lesions in fish will be developed. Groups of medaka (Oryzias latipes) have been exposed to either N-methyl-n-nitro-n-nitroso-guanidine (MNNG) or methylazoxymethanol acetate (MAM-Ac) to induce variable neoplasms and proliferative lesions to which IHC can be applied to identify differentiation antigens. Both peroxidase-antiperoxidase and avidin biotin complex techniques are used to identify intermediate filament proteins keratin, desmin, vimentin, glial fibrillary acidic protein, and neurofilament protein. Other selected antigens may also be tested for based on histology. These findings will be correlated with histology, and ultrastructure. The results of this proposal will identify associations between cell types in neoplasms and in proliferative lesions of uncertain origin which cannot yet be typed by morphology alone in the fish, and thereby identify the possible progression of these lesions

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I. Background

The increased prevalence of neoplasms in fish from chemically polluted bodies of water (10) has led to vigorous research efforts to determine what a tumor in a fish means from a human health perspective. One increasingly valid method of assessing human health risk is with the use of aquatic bioassays. Once established, bioassays using fish as alternative experimental animals can be utilized to test the toxic and carcinogenic potential of substances that may pollute water and food sources.

It originally appeared that in order to make aquatic bioassays valid alternatives to rodent toxicity and carcinogenicity testing, fitting the fish responses to injury as closely as possible to those seen in rodents was necessary. However, with my own experience in experimental research using fish and in consultation with Dr. Robert A. Squire, an expert in the area of determination of human cancer risk and key personnel on the current contract, it became evident that it was more important to 1) document each lesion produced with carcinogenic exposure, 2) determine if the lesion progresses or regresses (i.e., is the change permanent), 3) determine if the lesion progresses to a neoplasm (i.e., is it preneoplastic), 4) determine if the lesions are reproducible (predictable) with a repeat study, and most importantly, 5) determine the seriousness of the lesion in regards to the health of the fish (i.e., will it ultimately result in death, or produce no untoward effect).

In the original contract (DAMD17-88-C-8029, USABRDL), four groups of 14-day old medaka were exposed for 48 hours to 0, 100, 200, or 400 mg/L of diethylnitrosamine (DEN) and sequentially sacrificed at approximately 2, 4, 6, 8, 12, and 24 weeks post exposure to determine the effects of short term exposure protocols (DAMD17-88-C-8029) (4,5). Most of the lesions seen were

similar to those reported in bioassays using adults of various species of fish exposed to DEN, including foci of cellular alteration, cellular vacuolation, eosinophilic inclusions, and a variety of benign and malignant hepatic neoplasms. Both the incidence and severity of lesions produced, including malignancy, were directly related to exposure level. The neoplasms seen are shown in Table 1, and the results have been published (4,5).

In another study with USABRDL, medaka embryos (2 days prehatching age) were exposed to methylazoxymethanol acetate (MAM-Ac) for 4 hours at 20, 25, or 30°C followed by a grow-out period of up to one year. Numerous neoplasms developed in several organs although muscle tumors and other sarcomas seemed to predominate (Table 2). These data are to be submitted to Aquatic Toxicology (6).

However, in both the DEN and MAM-Ac studies at USABRDL, unusual proliferative populations of cells were seen in liver and soft tissues which were difficult to identify by morphologic criteria alone, and which have not been commonly seen in mammalian tissues. In addition, many neoplasms in the two studies were tentatively diagnosed (see *, Tables 1 and 2) because morphology alone was insufficient, and the knowledge about the origin and behavioral biology of certain cell types in the fish is currently inadequate.

Table 1. Total benign and malignant hepatic neoplasms

Type	Exposure group and total number of each tumor type
Adenoma	II (1), III (1)
Cholangioma	II (1)
Hepatocellular carcinoma	I (1), II (2)
Cystadenocarcinoma	II (1)
Cholangiocarcinoma	II (1), III (1)
*Sarcoma with vascular orientation	II (1), III (2)
*Histiocytic sarcoma	II (1), III (1)
*Carcinosarcoma	III (1)
*Malignant - undetermined type	II (1), III (1)

*Tentative based on histology and ultrastructure

Group I - 100 mg/L DEN; Group II - 200 mg/L DEN; Group III - 400 mg/L DEN.

Table 2. Types of neoplasms produced in the medaka with MAM-Ac embryo exposure (totals by end of the study)

Type	Tissue	Total number of each
Rhabdomyosarcoma	Skeletal muscle	11
*Leiomyosarcoma	Intestine	4
*Undifferentiated sarcoma	Skeletal muscle	5
*Undifferentiated sarcoma	Peripheral nerve	3
*Undifferentiated sarcoma	Peritoneum	1
Hepatocellular carcinoma	Liver	2
*Hemangiopericytoma	Liver	1
Cholangiocarcinoma	Liver	5
Adenoma	Liver	5
Adenocarcinoma	Swim bladder	2
Adenoma	Swim bladder	2
*Hemangiosarcoma	Gills	1
Papillary adenoma	Gall bladder	1
*Tentative diagnosis based on histology.		

It is imperative in the establishment of the fish bioassay that lesions be identified which may progress to form neoplasms that will compromise the host. One of the best ways to do this is by developing techniques such as immunohistochemistry, which can aid in the identification of cell types in proliferative, potentially preneoplastic lesions and in the neoplasms which may subsequently develop (2,3). In human and veterinary medicine and pathology it is exceedingly important to determine cell type in neoplastic disorders since the presence of one cell type can mean a good prognosis, while another can mean a poor one. With the development of immunohistochemical techniques that can be applied to paraffin embedded fish tissues, we will be able to determine tumor cell origin based on differentiation along a certain cell line, which will have broad applicability in the development of aquatic bioassays. These techniques are largely unavailable at the present time.

II. Body

A. Hypothesis and Significance

The majority of neoplasms, particularly well differentiated tumors, can be diagnosed by morphologic criteria. Bundles of spindle shaped cells are characteristic of sarcomas (mesenchymal origin), and glandular formation is indicative of carcinoma (epithelial origin). Within these broad groups, neoplasms can be further categorized by sets of morphologic criteria established through years of experience. However in undifferentiated tumors, often designated undifferentiated sarcoma or carcinoma, the neoplasms cannot be further classified. Even less differentiated neoplasms are sometimes given the designation "malignant tumor". Histochemical special stains have been used by pathologists to identify some features of differentiation such as the presence of certain lipids or enzymes. However, an additional powerful tool currently used in tumor diagnosis is immunohistochemistry (IHC), in which

FIGURE 1. Sample immunodiagnosis of a sarcoma

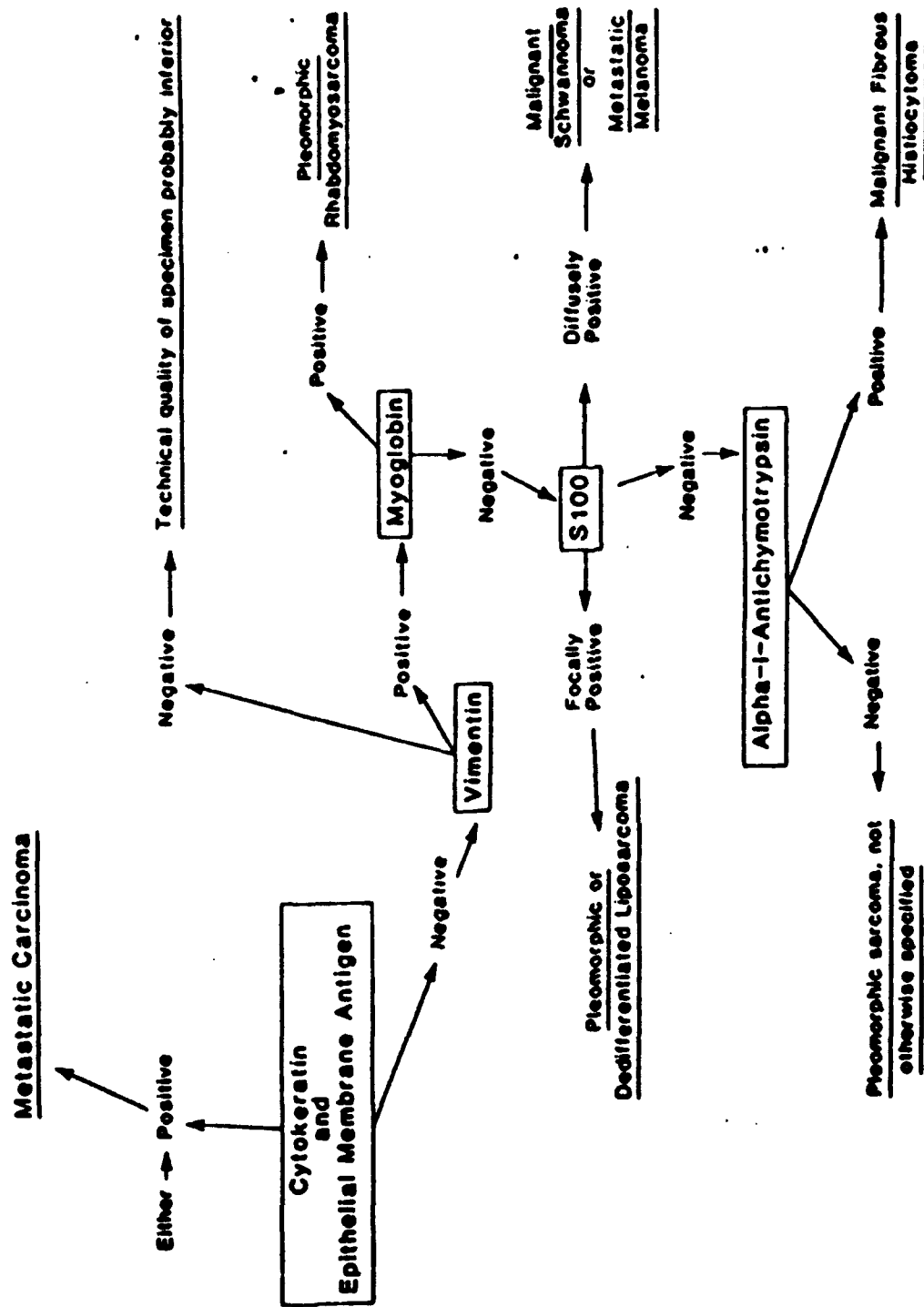


FIG. 19. Algorithm for immunodiagnosis of pleomorphic sarcomas of soft tissue.

From Wick, M.R., and Svensson, P.E., Soft Tissue Tumors. In Diagnostic Immunopathology. R.B. Colvin, A.K. Bhan, and R.T. McCluskey, eds. Raven Press, N.Y. 1988.

antibodies directed against certain "differentiation molecules" (substances expressed by tumor cells which indicate differentiation along a certain pathway) are used to locate these substances in tumor cells (1-3,8,9,11). In human pathology, IHC techniques are well developed and identification of numerous antigens in tumors is possible. The differentiation molecules include hormones (insulin, thyroglobulin, testosterone, etc.), intermediate filaments (keratin, desmin, vimentin, glial fibrillary acidic proteins neurofilament proteins), so-called tumor associated antigens (antigens produced by certain tumors) and many other substances based on molecules found in normal cell types (i.e., alpha-1-antitrypsin in liver, factor VIII in endothelial cells). By using a panel of monoclonal antibodies on a tumor, the presence and/or absence of certain antigens identifies the tumor cell lineage along a certain differentiation pathway (1-3,8,9,11). An example of immunodiagnosis of an undifferentiated sarcoma is shown in Figure 1 (12). Immunohistochemistry must always be used in conjunction with morphology to complement a morphologic diagnosis, and not as a replacement. Generally, monoclonal antibodies produced by hybridoma cell lines are utilized in IHC rather than polyclonal antibodies due to increased antigenic specificity and decreased variability (1).

The hypothesis for this study is that the development of immunohistochemical methods for the identification of carcinogen induced neoplasms and other proliferative cellular lesions of unknown origin from paraffin embedded fish tissues will contribute significantly to the establishment of aquatic bioassays as valid, reliable alternatives in carcinogenicity and toxicity testing to assess human health risk. The development of additional diagnostic tests such as IHC are important because 1) fish tissue can respond to injury in ways atypical to mammals by the

formation of unusual proliferative or neoplastic lesions which cannot yet be diagnosed by morphologic criteria alone, 2) these atypical lesions must be identified to determine the possible progression of these lesions to various types of neoplasms; a critical factor in determining the health effects or risks of a compound, 3) IHC methods have the unique ability to allow direct correlation of histologically detected lesions with their antigenic expression, and 4) these techniques are largely unavailable at the present time, so that this body of work is unique.

The continuation of this contract also highlights the concern of the military with environmental issues. There is enormous interest in this area of research from the scientific and public sectors as environmental pollution is finally a topic of world-wide concern. This is even more critical for field military personnel as their exposure risk to contaminated water and/or food supplies can become considerable. The development of a small fish aquatic bioassay is important from a military standpoint because once the database is established, the aquatic bioassay 1) may provide a more rapid, less expensive alternative to rodent toxicity and carcinogenicity testing for determining military personnel health risk to noxious compounds that might be generated by military operational procedures or are present in the field from other sources, and 2) may allow determination of the effects of these potentially noxious compounds on aquatic ecosystems. These are exceedingly important goals from public, environmental, and military standpoints.

B. Methods

Two groups of medaka have been exposed to MAM-Ac or N-methyl-n-nitro-n-nitroso-guanidine (MNNG) to induce a variety of neoplasms. The fish upon sacrifice are placed in Bouins fixative with small portions of liver or other grossly detectable neoplasms placed in 3.0% glutaraldehyde in 0.1M

cacodylate buffer for electron microscopy.

The intermediate filament proteins were chosen to develop first as they have demonstrated phylogenetic preservation in several species, and as they are most commonly used in tumor diagnosis (Table 3). Other antigens may be tested later as shown on Table 3. The IHC method primarily used is the streptavidin-biotin-peroxidase method (7) shown in figure 2. For some antigens, the peroxidase-antiperoxidase method may be necessary (figure 3). For the preliminary studies, tissues were also taken from striped bass (Morone saxatilis) for comparison purposes. To date, two keratin antibodies have been extensively tested on normal medaka and striped bass tissues and results are as follows.

C. Results

The results of the application of AE1/AE3 (Boehringer Mannheim, Indianapolis, IN) and MAK-6 (Triton Biosciences, Alameda, CA) to fish tissues are shown in Tables 4 and 5. In general, the fish and mammalian tissue responses to the antibodies were similar. In striped bass, both antibodies showed strong positivity in skin, gills, cornea, renal tubules, the gastrointestinal tract, simple ducts associated with pancreatic acini, bile ducts through their terminal divisions, and thymic Hassall's corpuscles. In general, Bouins fixative improved the results seen compared to formalin fixation.

In the medaka, skin, gills, cornea, and upper gastrointestinal tract were similarly reactive to both antibodies as seen with striped bass. However, renal tubules and bile ducts stained less intensely or not at all. A problem with adequate fixation cannot be entirely ruled out, although several methods of tissue dissection (sagittal section, transverse section, and individual organ removal) were tried in preliminary studies without significant

Table 3. Possible differentiation antigens to be labeled in neoplasms from the medaka

Antigen	Application
Intermediate filament proteins:	
*Keratin (19+ different proteins 40-60KD)	Detects epithelial differentiation (carcinomas)
Vimentin (1 protein 58KD)	Detects mesenchymal differentiation (sarcomas)
Desmin (1 protein 53KD)	Detects smooth and skeletal muscle differentiation
Glial fibrillary acidic protein (GFAP - 1 protein 51 KD)	Detects glial cell differentiation
Neurofilament (3 proteins 68, 150, and 200 KD)	Detects neuronal cell origin
Other:	
Alpha-1 antitrypsin	Common marker for hepatocellular carcinoma
Alpha-1 antichymotrypsin	Marker for histiocytic differentiation i.e., malignant fibrous
histiocytoma	
Factor VIII/UEA-1	Markers for endothelial cell differentiation
Myoglobin	Skeletal muscle differentiation i.e. rhabdomyosarcoma
Epithelial membrane antigen (EMA)	Detects epithelial differentiation
S-100 protein	Marker for nerve sheath tumors, melanoma

***Different keratins can be used to detect different types of epithelium, i.e. to differentiate hepatocellular carcinoma from cholangiocellular carcinoma (8).**

FIGURE 2. Avidin-Biotin-Peroxidase Complex Technique. Modified from A.K.Bhan, Immunoperoxidase, in Diagnostic Immunopathology

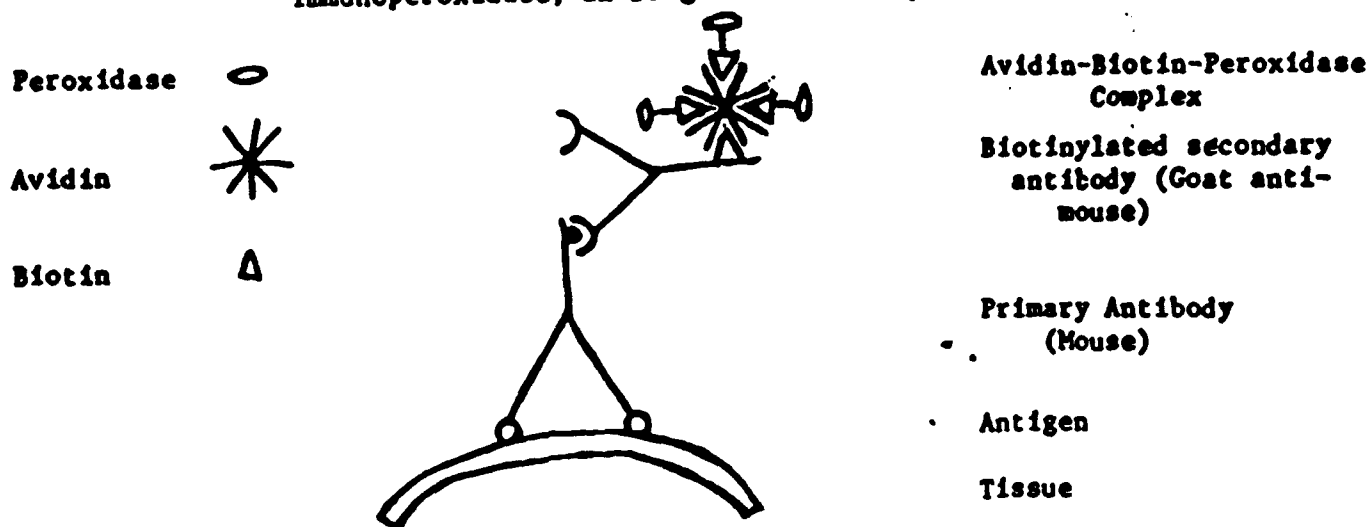


FIGURE 3. Peroxidase antiperoxidase technique. Modified from A.K. Bhan, Immunoperoxidase, in Diagnostic Immunopathology

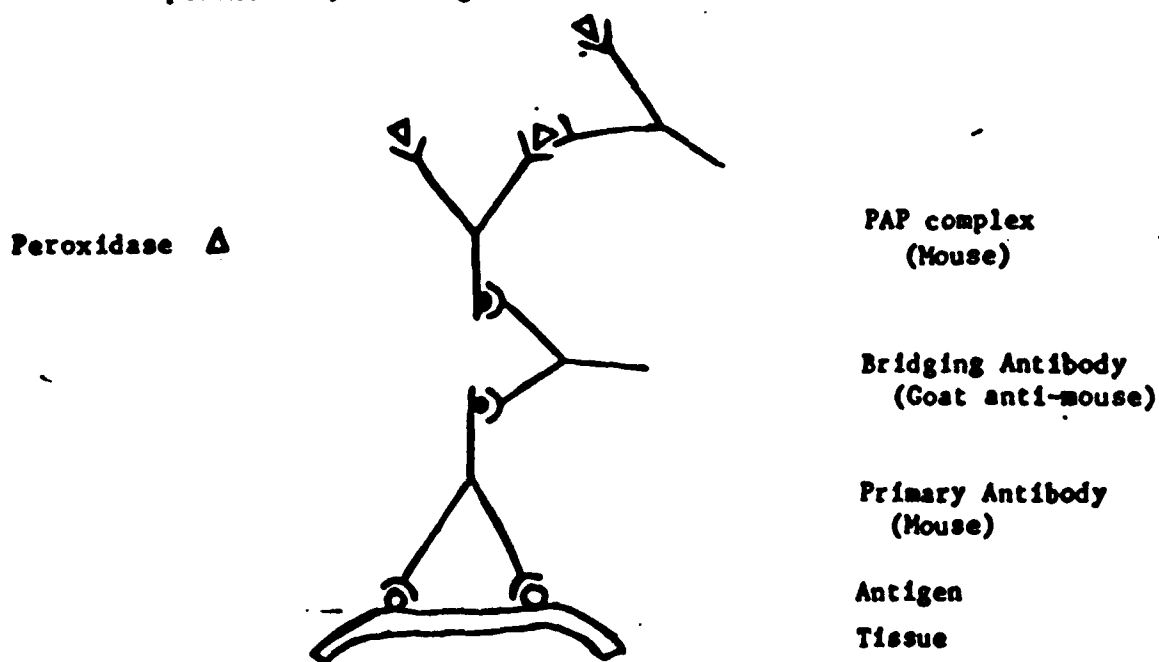


Table 4. Cytokeratin reactivity in striped bass relative to antibody and fixative

<u>Tissue</u>	<u>Bouins Fixative</u>		<u>Formalin Fixative</u>	
	<u>AE1/AE3</u>	<u>MAK-6</u>	<u>AE1/AE3</u>	<u>MAK-6</u>
Skin	+++	+++	+++	+++
Gills	+++	+++	+ / ++	+ / ++
Eye				
Cornea	+++	+++	++	+++
Retina	-	-	-	-
Liver				
Hepatocytes	-	-	-	-
Bile ducts	+++	++	+	- / +
Kidney				
Tubules	+++	++	++	++
Glomeruli	-	-	-	-
Gastrointestinal tract				
Oral cavity	+++	+++	+++	+++
Esophagus	+++	+++	+++	++
Stomach	+++	++	+++	+
Intestine	+++	++	++	+
Pancreas				
Acini	-	-	-	-
Islets	-	-	-	-
Brain/spinal cord	-	-	-	-
Spleen	-	-	-	-
Head kidney	-	-	-	-
Thymus				
Lymphocytes	-	-	-	-
Hassall's corpuscles	+++	+++	NP	NP
Muscle	-	-	-	-
Heart	-	-	-	-
Bone/cartilage	-	-	-	-
Ovary/Testis	-	-	-	-

+++ = strong; ++ = moderate; + = fair, - / + = weak and patchy
 NP = tissue not present on the slides examined

Table 5. Cytokeratin reactivity in medaka relative to antibody and fixative

<u>Tissue</u>	<u>Bouins's Fixative</u>		<u>Formalin Fixative</u>	
	<u>AE1/AE3</u>	<u>MAK-6</u>	<u>AE1/AE3</u>	<u>MAK-6</u>
Skin	+++	+++	++	++
Gills	+++	+++	+++	+ / ++
Eye				
Cornea	+++	+++	++	+++
Retina	-	-	-	-
Liver				
Hepatocytes	-	-	-	-
Bile ducts	+	-	- / +	-
Kidney				
Tubules	+	+ / ++	- / +	+ / ++
Glomeruli	-	-	-	-
Urinary bladder	+++	++	+	- / +
Gastrointestinal tract				
Oral cavity	+++	+++	+++	++
Esophagus	+++	+++	+ / ++	++
Intestine	-	-	-	-
Pancreas				
Acini	-	-	-	-
Islets	-	-	-	-
Brain/spinal cord	-	-	-	-
Spleen	-	-	-	-
Thymus				
Lymphoid cells	-	-	-	-
Hassalls corpuscles	+++	++	-	NP
Muscle	-	-	-	-
Heart	-	-	-	-
Bone/cartilage	-	-	-	-
Ovary/Testis	-	-	-	-
Oviduct	+	-	-	-
Thyroid	-	-	-	-

+++ = strong, ++ = moderate, + = fair, - / + = weak and patchy

NP = tissue not present on the slides examined.

differences in results. Another striking difference from striped bass was the poor staining of intestinal epithelium. The positive cytokeratin staining ended abruptly at the level of the intestine (the esophagus joins the intestine in the medaka), although weak, patchy staining of the intestinal epithelium was sometimes seen. This may indicate a difference between the two species of fish in the types or amounts of cytokeratins present in the intestine.

Good results have also been produced using anti-GFAP and neurofilament antibodies on fish tissues, producing typical antigenic responses as seen in mammals. Anti-desmin antibodies have produced less than optimal results possibly because the protein structure is not preserved between these species.

Preliminary results on the application of anti-keratin antibodies to neoplasms produced by MAM-Ac in the medaka have shown that biliary neoplasms are positive, while hepatocellular neoplasms are negative as would be expected.

III. Conclusions

The results to date show that there is good phylogenetic preservation of intermediate filament antigens and that antibodies can be usefully applied to paraffin embedded fish tissues. These results will expand enormously the potential of the aquatic bioassay. There is considerable work to be done however in working up each antibody properly as it is time intensive. Future work will concentrate on antibodies which detect the presence of sarcomas, such as vimentin, factor VIII, etc.

The streptavidin-HRP method used is simple, direct, and produced clear, reproducible results. Although investigators will need to develop independent standards for each species of fish, the broad cross reactivity seen between these two divergent species of fish and the mammalian controls is indicative

of the potential for use of this technique as a diagnostic tool in fish carcinogenesis research.

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